

# The role of crop-pollinator relationships in breeding for pollinator-friendly legumes: from a breeding perspective

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**Abstract** Breeders are encouraged to develop breeding approaches that strive to integrate food production into the healthy functioning of agro-ecosystems. In the case of legumes, this approach should preserve bee fauna by providing suitable floral resources within the crops themselves. In parallel, legume breeding for sustainable agriculture is linked to the development of environmental services. Foraging places and nesting sites for solitary and social bees are some of the ecological services provided for legumes. Crops with floral attractiveness and rewards for insects can be used

to enhance pollinator conservation as well as crop yield and yield stability. We analyze how understanding crop-pollinator relationships (CPR) can contribute to the production of high-yielding and pollinator-friendly varieties by examining: (1) The status of knowledge on mating systems and floral traits; (2) The contribution of CPR understanding to plant breeding for both hybrid-seed production and open-pollinated population improvement.

**Keywords** Bee pollinator · Floral traits · Heterosis · Hybrid and population improvement · Soybean · Faba bean

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## Introduction

Biologists recently compiled a world check list of bees. Nearly 19,500 bee species were identified on six continents (Integrated Taxonomic Information System (ITIS), <http://www.itis.gov/beechecklist.html>). Scientists have estimated that the worldwide economic value of the pollination services provided by insect pollinators in 2005 was about €153 billion (Gallai et al. 2009). This figure amounted to 9.5% of the total value of world agricultural food production. Furthermore, the value per hectare of crops that depend on insect pollinators for their production was on average much higher than that of crops not pollinated by insects. There was a positive correlation

between the value of a crop category per production unit and its ratio of vulnerability based on its dependence on insect pollinators.

Climate changes exacerbate concerns about agricultural production and food security worldwide (Rosenzweig and Parry 1994; Chmielewski et al. 2004). At the global level, ecosystems are under threat; both productivity and geographic distribution of crop species will be affected. This, in turn, will affect insect pollinators and plant pollination. For example, air pollution can lead to reductions in the concentration of volatile compounds that insects detect as they forage for nectar and pollen (McFrederick et al. 2008). Faba bean plants growing under elevated CO<sub>2</sub> conditions showed an increased floral display and may be more attractive to pollinators, but pollen flow may not necessarily be improved (Osborne et al. 1997).

Changes in crop management techniques, particularly the intensification of cropping, reduction/change in crop rotations, and increases in monocultures, have intensified the activity of pests (Rosenzweig et al. 2002). The expansion of world trade in food and plant products has increased the impact of weeds, insects, and diseases on crops. International movement of insect pollinators, and their associated pests, was offered as a possible contributor to colony collapse disorder (CCD) of honeybee colonies in the US.

Adaptive modifications initiated by agriculturists, horticulturists, and foresters can help to minimize the negative impacts of a decline in insect pollinators. An example is the recent trend in sustainable agriculture for environmental priorities to be incorporated into plant breeding objectives (Mena et al. 2005). Following reports of declines in solitary and social bees, breeders were encouraged to develop breeding approaches that strive to integrate food production into the healthy functioning of agro-ecosystems (Allen-Wardell et al. 1998; Maria-Klein et al. 2007). In the particular case of legumes, this approach could help preserve and enhance bee fauna by providing suitable floral resources within the crops themselves.

In parallel, legume breeding for sustainable agriculture is linked to the development of non-food services such as environmental services (Helenius and Stoddard 2007). Legume improvement demands a shift in emphasis in breeding programmes from yield alone towards additional emphasis on environmental function. Legumes are visited by a great

number of social, eusocial, and solitary bees, which can be commercial, feral, or wild (Free 1993; Delaplane and Mayer 2000). Foraging places and nesting sites for solitary and social bees are some of the ecological services provided by legumes in a sustainable agriculture. The provision of floral resources within the crop for supporting beneficial insect pollinator populations could be a promising strategy to enhance environmental function of legumes. Consequently, we face a situation where the development of pollinator-friendly cultivars is needed. Such a situation may require a re-thinking of crop breeding strategies and objectives.

The role of bee pollinators as agents of pollination and of hybridisation needs to be considered. Pollinators help increase seed set and self-pollination, but more importantly enhance cross-pollination (Richards 2001). Pollinators as agents of cross-pollination can contribute to breeding strategies. Crops with suitable floral attractiveness and rewards can be used to foster pollinator conservation, which in turn may lead to improved seed set and/or improved crop yields as well as increased resistance to biotic and abiotic stresses.

There are good breeding reasons as well as ecological reasons for improved targeting of crop-pollinator relationships (CPR) (Westerkamp and Gottsberger 2000; Aizen et al. 2008). Optimising CPR would be a key to the establishment of breeding strategies that increase yield and its stability by using social and solitary bees, thereby facilitating the development of “pollinator-friendly” cultivars. The beauty of this breeding strategy is that farmers might get additional income from conservation payments, and simultaneously from conserving biodiversity (Morandin and Winston 2006). And the farmer would still continue to get high yield and yield stability.

The benefits of approaching legume improvement by applying an understanding of CPR are both direct (seed yield and stability increase) and indirect (conservation of biodiversity and beneficial insects). To make effective progress in breeding for pollinator-friendly varieties, there are two complementary aspects of breeding that need consideration. Firstly, breeding efforts and technologies should carefully match plant and pollinator requirements; yield, yield stability, and resistance to stresses on one hand and pollinator needs on the other. Secondly, these varieties may require the introduction of new traits or the enhanced expression of existing traits to optimize CPR.

How can the understanding of CPR contribute to underpinning the production of pollinator-friendly varieties? The ultimate goal of CPR understanding, from the viewpoint of plant breeding, is the development of an assisted crossing strategy for exploiting agronomic performance, e.g. heterosis.

### The contribution of CPR understanding to plant breeding

An understanding of CPR can provide potential tools to assist breeding methodologies, while at the same time preserving bee populations by developing specifically adapted cultivars. For this review, we will focus on legume improvement, both for hybrids and populations. Although CPR understanding is also relevant to “on farm” and “in situ” conservation methodologies (Horneburg 2006), these are not considered in this paper.

Demonstration of heterosis for yield and other valuable traits in several legumes including pigeon-pea (Saxena 2006), and alfalfa (Paul Sun, Dairyland Seed Co. personal communication, 19 November 2008) has prompted efforts to consider hybrids in other legumes, such as soybean (Palmer et al. 2001). Although it should be noted that heterosis can also operate in open-pollinated populations (Ebmeyer and Stelling 1994; Stelling et al. 1994). Any increase that can be made in the proportion of hybrids in open-pollinated or synthetic populations with mixed breeding systems should increase the yield potential (Gasim and Link 2007).

However, heterosis is only one of several agronomic performance determinants for the success of hybridization programs (Duvick 1999). For instance, the primary barrier for commercial use of hybrid soybean is the lack of an economical method of seed production because of limited pollen transfer by insects (Palmer et al. 2001). Efforts to exploit heterosis in self-pollinated or partially allogamous legumes require a search for both plant and pollinator traits that contribute to out-crossing.

Thus, there is a major application-oriented question for heterosis exploitation in legume plant breeding that deserves attention. Are there efficient and practical cross-pollination systems available that are yet to be identified? A better understanding of CPR could help resolve this question, by offering

alternative methods to manipulate pollen dispersal and receipt, and ultimately lead to better out-crossing systems (Davis 2001).

The aspects of CPR relevant for breeding will vary depending on the objective—hybrid or population improvement. Hybrid programs should focus on matching both male and female sexual traits in the targeted environments where hybrid-seed production is to occur. Population improvement or recurrent selection methods should focus on traits correlated with high levels of allogamy (Lewers and Palmer 1997). An efficient and cost effective hybrid program needs to target basic questions regarding the matching of female and male traits and consider traits related to both pollen production and transfer, and to female function (Tregenza and Wedell 2000; Harder et al. 2004).

Patterns of pollinator visitation may limit crossing among female and male plants. Pollinator foraging, and thus pollen transfer, may occur only rarely because the male and female parents have different pollination syndromes (Willson 1994; Ashman et al. 2005). However, hybrids can be obtained between the male and female when they have compatible floral phenotypes and an appropriate pollinator(s). In breeding research, knowledge of the environmental and genetic factors associated with CPR are relevant because these factors will help determine the optimal combinations of floral phenotypes and effective pollinators.

A different approach to apply CPR to the exploitation of heterosis could be advocated for population improvement. Population improvement through recurrent selection involves several steps: developing a broad-based population, selecting and evaluating plants or families and intermating the best plants or families to constitute the next population. The methodological question that arises within this process is: How to elevate out-crossing to increase heterozygosity and recombination potential (Barrett 2008)? Out-crossing, and consequently the level of heterozygosity, would be increased through selection for floral characteristics that enhance its frequency (Abdel-Ghani et al. 2003, 2004, 2005; Suso et al. 2005a; Parzies et al. 2008; Singh et al. 2007). More information on ways in which floral traits influence mating systems is needed for legumes. Appropriate pollinator visitation greatly affects the mating system. Pollinator choice is determined first by attraction and

subsequently by presence of rewards. Floral traits can be used by potential insect visitors as cues to the value of the reward in a given plant. When pollinators associate specific floral traits with increased rewards, they are more likely to continue to forage on plants with those traits (Widrlechner and Senechal 1992). Consequently, our understanding of CPR offers additional options to increase out-crossing in breeding for heterosis exploitation in populations while simultaneously fostering bee conservation.

### The status of knowledge on mating systems

CPR is not a topic that readily comes to mind when grain legumes are being considered. One common view of grain legumes is that they display autogamous or mostly autogamous mating systems (Suso et al. 2005b). The shift from out-crossing or facultative selfing to strict inbreeding has been described as the single most common trend in legume domestication (Rick 1988). Inbreeding favors the evolution of plants with lower levels of insect pollinator attraction and reward (Charlesworth 2006). Plants that produce less nectar and pollen often exhibit less pollinator visitation and, consequently, give less support for pollinator conservation. Nevertheless, though the majority of the grain legumes are considered self-pollinated, they possess perfect flowers capable of out-crossing (Suso et al. 2005b).

Before going further, it is instructive to reflect on empirical methods to describe mating systems. A common descriptor of the mating system is the estimated out-crossing rate (the proportion of offspring fathered by genetic individuals other than their seed parent; Neal and Anderson 2005). The accuracy of that rate and, thus, our understanding of the mating system depends on the availability of markers, the precision and accuracy of their measurement, and methodologies used to process the data generated. Different statistical approaches are available to assess the degree of cross-fertilization (Jain 1979; Ritland and Jain 1981; Enjalbert and David 2000; Ritland 2002). Hence, it is valuable to reflect on what we know from each approach because sometimes the biological meaning of different estimates vary widely that direct comparisons are not valid. Historically knowledge of mating systems was based on controlled pollination and statistical approaches based on the classical tools of

visual markers, such as heritable differences in floral pigmentation. However, the use of pigment polymorphisms is problematic, because of differences in how various pollinators process and apply colour-related information (Leleji 1973; Vries 1978; Stanton et al. 1989; Steiner et al. 1992; Rahman et al. 1995).

Procedures used to estimate the degree of cross-pollination in classical marker studies refer to the frequency of hybrids that would have resulted from crossing between pollen donor plants (male function) with the dominant marker and the pollen recipients (female function) with the recessive marker; that is inter-genotypes crossing (Tayyar et al. 1996; Horneburg 2006; Toker et al. 2006; Ferreira et al. 2007).

Molecular markers with high allelic variation, such as simple sequence repeats (SSR), and more elaborate biometrical models facilitated the development of multilocus approaches (Ritland 1990, 2002). Multilocus approaches can use information from all genotypic categories and numerous loci, and thus more accurately reflect the total amount of out-crossing in open-pollinated populations (Escalante et al. 1994; Yagoubi and Chriki 2000).

In recent decades, our understanding of mating systems has undergone fundamental, conceptual changes. Initially, there had been a general acceptance of selfing and out-crossing as two relatively symmetrical and evolutionarily stable states. Populations of plants were thought of as completely outbreeding or completely inbreeding. However, recent studies (Vogler and Kalisz 2001) demonstrated that animal-pollinated plant populations often present a mixed mating system. For example, 42% of insect-pollinated plant species surveyed to date have intermediate levels of out-crossing. It is now clear that insect-pollinated plants display a dramatic range of mating systems, but mixed mating systems are becoming recognized as the norm, rather than the exception (Goodwillie et al. 2005; Charlesworth 2006).

Despite rapid progress in genetic markers, we are still some way from understanding for any population the basis of mating information of the proportion of offspring fathered by genetic individuals other than the seed parent, because most mating system studies to date have adopted a largely inter-crossing and morphological approach (Chowdhury and Slinkard 1997; Ferreira et al. 2000, 2007; Toker et al. 2006).

Mating systems are complex traits. Breeders should focus more attention on understanding the

factors controlling mating systems. Both genetic and environmental influences are important: the relative contribution of genetic and environmental factors determine the potential of response to selection by breeders interested in mating-system traits. For example, genetic variation for floral traits influencing the breeding system was demonstrated in the wild tomato relative, *Solanum pimpinellifolium* (Georgiady et al. 2002; Georgiady and Lord 2002), and there is evidence that this variation is geographically structured (Rick et al. 1977, 1978; Widrechner 1987), and that its heritability may be high (van Kleunen and Ritland 2004). The potential for changes by selection can be considerable (Bixby and Levin 1996; Horneburg 2006).

To what extent can mating systems be altered by selection? What reproductive characters change as correlated responses to selection for out-crossing?

### Key aspects of floral traits for improving CPR

To optimize the balance between high yielding varieties and those that support pollinator conservation, the identification of desirable floral phenotypes is crucial. It is necessary to focus on traits associated with CPR, such as floral attractiveness, in terms of colour, design, display, and phenology, and rewards, both of pollen and nectar, which are often beyond the objectives of most legume improvement programs.

Bees require plants for foraging and nesting sites and may also visit flowers to satisfy other idiosyncratic behavioural requirements. They try to make their living feeding on nectar and/or pollen, seeking shelter, and reproducing (Westerkamp and Weber 1999; Kudo and Harder 2005). As flowers present signalling characters subject to pollinator interpretations, bee foraging patterns are very much influenced by floral characters and plant spatial patterns (Raguso 2004). Floral characters can function with respect to pollinators either by affecting their behaviour, for instance attracting them with larger petals or with nectar of a different chemical composition (Carter et al. 2006), or by modifying the physical contact between the pollinators and the anthers or stigma (Chess et al. 2008).

How do floral traits influence bee behaviour? The reason why an insect forages on a particular flower can be partly attributed to differences in floral design

and floral display (Harder and Barrett 1996; Goulson 1999). Floral display describes the number of flowers open at one time and their arrangement in inflorescences; whereas, floral design refers to characteristics of individual flowers including their morphology, colour, scent, nectar quantity and composition, and pollen production (Barrett and Harder 1996).

The morphology of legume flowers is often complex, with each component serving a specific function. For those taxa that rely on exogenous pollinations, the floral components attract pollinators and then channel them past the anthers and stigma where pollen and nectar are dispatched and received, often with exact precision (Westerkamp and Weber 1999). There is evidence that supports the view that floral morphology is actively selecting for mechanical fit, pollinator strength, and concerted patterns of behaviour in determining the frequency of visits and the allocation of rewards (Harder 1985; Wilson et al. 2004).

Ollerton and Dafni (2005) consider the functional morphology of extra-floral and floral organs; those functions most pertinent to legumes are listed in Table 1. A typical legume flower comprises a pattern consisting of sepals, and five petals, including one standard, two wings, and two keels, stamens and carpels. In addition, extra floral organs, such as stipule extra-floral nectaries may also play a role in pollinator attraction (Westerkamp and Weber 1999).

Appropriate understanding of floral function is a suitable tool for assisted crossing technologies for exploiting heterosis through breeding. However, the heritability of nectary attributes has received limited attention (Mitchell 2004). For example, analysis of the floral function could help in the identification of major traits associated with out-crossing that could be utilized for population improvement. Out-crossing may be the result of genes modifying the size of the standard petal and/or decreasing the production of nectar. Furthermore, male function success in delivery of pollen to receptive stigma has a critical role in hybrid breeding. Selection for traits that increase the male behaviour depends on attraction of pollinator visits and the effectiveness of pollen transfer in each visit which in turn depends on, for instance, the specific arrangement of ovary and style and the secondary pollen presentation for appropriate pollinator fit. However, to breeders, many of the floral traits discussed here may at first glance seem to be of little relevance. Yet every individual CPR, due to its

**Table 1** Functional morphology of extra-floral and floral organs in relation to pollination

Organ	Function
Stipule extra-floral nectaries	Protection Advertisement—visual and olfactory Temporary retention of pollinators
Calyx	Protection of the whole flower when closed and young buds prior to flowering
Corolla (standard, wings and keel petals fit together in a precise manner by which the flower forms a unit with a mechanism well adapted to bee pollination)	Advertisement Long range: visual, colour, position size, inflorescences, organization Close range: visual, nectar guides Signalling Flower colour Direction of pollinator behaviour (spatial orientation) Restricting diversity of pollinators Isolation of reproductive organs
Filament and anther	Protection Protection of nectar of the female organs by creating a tube around them Reproduction Pollen production Reward Alternative reward for nectar for pollen-collecting bee
Stigma	Pollen reception
Style	Pollen reception Spatial positioning of the stigma to allow efficient pollination Secondary pollen presentation Direction of pollinators Selection of pollinators
Ovary	Reproduction Bearing ovules Reward Bearing nectaries

Source: Ollerton and Dafni (2005); Westerkamp and Weber (1999)

influence on the visit made by the bee pollinator, has important consequences for legume breeding.

Davis (2001) summarized floral traits and nectar characteristics that are applicable to plant breeding programmes. Progress has been made through breeding to improve honey-production potential (Shuel 1989), and in nectar production (Barnes and Furgala 1978; Campbell and Ayers 1989). Breeding for floral structural features has resulted in changes in nectar yield. For example, flower size, floral biomass, size of the corolla, length of the gynoecium, flower stalk (peduncle cross-sectional area and vascular supply) have shown response to selection (reviewed by Davis 2001).

The receptacle base (receptacle size) has received attention in selection for nectar production. In the legume *Lotus corniculatus*, the best indication of nectar carbohydrate per floret was receptacle height (Campbell and Ayers 1989). However, with other *Lotus* species, nectar sugar quantity was correlated with receptacle volume (Campbell and Ayers 1989). In alfalfa, *Medicago sativa*, there were positive correlations between nectar volume and diameter of the receptacle (Teuber et al. 1983, 1990). In alfalfa, germplasm pools have been developed by phenotypic recurrent selection for; (1) ease of floret tripping (Knapp et al. 1996); (2) nectar volume (Teuber and Green 1996a); and (3) receptacle diameter (Teuber



and Green 1996b). These germplasm pools should provide the germplasm to study the effect of environment by genotype interactions for these three very important traits.

In recent years, the tools and methods available to reproductive biologists have changed significantly (Dafni et al. 2005). For instance, until recently, most floral morphological analyses consisted of time-consuming manual measurements. Now researchers can use digital image analyses systems (DIAS) that can accelerate phenotypic scoring. Morphological traits can also be quantitatively scored by electronic means rather than through the use of mechanical instruments. For instance, floral organs can be photographed or scanned and their size and shape analyzed by using various image software packages. DIAS of traits, such as standard length, width, area, and perimeter, facilitates extensive and detailed phenotypic analyses to describe subtle differences in flower size (Yoshioka et al. 2004, 2005). Appropriate software packages can provide more accurate, consistent, and objective measurement for floral traits, such as style length, ovary style angle (for assessment of the mechanical fit to pollinator) or pollen presentation that were impossible or impractical to determine manually (Suso et al. 2005a).

Previously, pollen counts could only be made painstakingly with a microscope; now they can be done much more quickly with an electronic particle counter (Harder 1998). Nectar concentration can be measured easily in the field with hand-held refractometers. Floral display, in terms of the volume of an inflorescence, can be determined with a 3-D space digitizer. The 3-D digitizer can provide a valuable method for describing the three dimensions of inflorescence architecture. The 3-D digitizer is now a routine biological technique (Friedman and Harder 2005) and could be useful for plant breeders to replace tedious, manual characterization.

Most flowering legume plants display multiple flowers, so that interactions with pollinators may vary with both the characteristics of individual flowers and with the aggregate properties of the entire floral display, especially the number of open flowers at any one time. These effects are seldom examined in concert and their collective consequences for pollen transfer or out-crossing are not usually considered.

Novel reproductive biology technologies, if accompanied by well-crafted descriptive models and

other analytical tools should help to enhance CPR understanding. The empirical basis of research on CPR is formed from various types of observations, including those on pollinator behaviour and diversity, mating-system estimations, and floral traits. These observations can often be organized into large matrices that can then be analysed by a range of widely available multivariate analysis programs, such as principal-component, multiple-regression and path analyses (Ollerton and Dafni 2005). Resulting products can include a set of descriptive statistics along with graphical plots that help describe CPR.

## CPR analysis in two legume taxa

### Glycine

#### *Soybean flowers*

Soybean has a typical papilionaceous flower with a tubular calyx of five unequal sepal lobes and a five-member corolla that consists of a posterior standard petal, two lateral wing petals, and two anterior keel petals (Guard 1931). The androecium consists of 10 diadelphous stamens in which the filaments of nine of the stamens are fused, leaving the posterior stamen separate. The single pistil is unicarpellate and has one to four campylotropous ovules. The style curves back toward the posterior stamen and is surrounded by a knoblike stigma that is receptive to pollen at anthesis (Carlson and Lersten 1987).

Soybean flowers open early in the morning, depending upon weather conditions. Pollen normally is shed shortly before or shortly after anthesis and typically effects self-pollination. Pollen germination usually occurs within 15–30 min after its placement on the stigmatic surface (Gordienko 1977). An artificial cross-pollination technique for soybean has been described (Paschal 1976; Fehr 1980). Successful pollinations were increased with higher relative humidities in Australia (Byth 1966). Since the female reproductive organs are usually mature about 1 day before the male (Carlson and Lersten 1987), it is not necessary to emasculate before making artificial cross-pollinations when pollinations are well timed.

In spite of facultative autogamy, soybean flowers possess most, if not all, of the anatomical adaptation characteristics of entomophilous plant species

(Juliano 1976; Erickson and Garment 1979; Arroyo 1981; Erickson 1983; Delaplane and Mayer 2000). Soybean floral nectaries are small, highly developed, and surround the carpel base. These are heteromorphic structures that produce fluids and/or volatiles that can attract insects (Horner et al. 2003; Table 2). The soybean flower also has well-defined visible and ultraviolet nectar guides, a tongue channel, tongue guides, and a nectary ridge that contains stomates that exude volatiles (Erickson 1979; Erickson and Garment 1979; Horner et al. 2003). Honeybees can be attracted to soybean flowers and recognize them by their colour, shape, and volatiles (Erickson 1976, 1983, 1984; Severson and Erickson 1984)).

Chiang and Kiang (1987) determined the number of pollen grains per ovule and classified soybean as falling between the classes of facultative allogamy and allogamy (Cruden 1977). Palmer et al. (1978) reported a range of 3,740–7,600 pollen grains per flower among five cultivated soybean lines grown in three environments. Fujita et al. (1997) had, on average, about 2,000 pollen grains per wild annual soybean (*G. soja* Siebold & Zucc.) flower. Because soybean is classified between Cruden's (1977) facultative allogamy and allogamy classes, it is plausible to assume that soybean probably shifted its breeding habit from out-crossing to highly selfing in recent evolutionary history (Arroyo 1981; Erickson 1984; Chiang and Kiang 1987; Kiang and Chiang 1989). Insects that prefer pollen rather than nectar might discriminate among soybean genotypes based on pollen quantity and/or quality. It is not known if breeding for larger soybean anthers (more pollen grains per anther) of a pollen parent, and coupled with insect-mediated cross-pollination, would result in increased seed-set on male-sterile plants. Geometric manipulation of different genotypes in high-density plantings that flower synchronously and are rich in nectar and pollen should favour insect visitation (Chiang and Kiang 1987). Thus it should be possible to increase the rate of out-crossing and seed yield on male-sterile, female-fertile soybean plants using such planting patterns.

#### Natural cross-pollination

**Wild Annual and Perennial Species.** The wild annual soybean, *G. soja*, is believed to be predominantly self-pollinated, however, little effort has been made

to evaluate its breeding system except by examining the number of polymorphic loci as revealed by allozyme variation (Bult and Kiang 1992; Kiang et al. 1992; Yu and Kiang 1993; Pei et al. 1998). Kiang et al. (1992) estimated an out-crossing rate of 2.3%. Fujita et al. (1997) analyzed the genetic structure of four *G. soja* populations in Japan by examining allozyme variation. They obtained higher within-population genetic variation and lower genetic divergence among populations than would be expected for a selfing plant species. The mean out-crossing rate estimate was 13%, ranging from 9.3 to 19% among the four populations. This higher out-crossing rate was supported by observations of frequent visits during flowering by honeybees and carpenter bees.

Field studies in Japan have shown that where *G. max* and *G. soja* are sympatric, their flowering periods overlap. Individual off-type plants among the wild annual population were hybrid derivatives of *G. soja* × *G. max* (Kaga et al. 2005; Kuroda et al. 2005). Similarly, Kwon (1972) reported natural hybrids in South Korea between *G. soja* and *G. max*. Kuroda et al. (2008) used seven locations (14 populations of *G. soja*) in northern, central, and southern regions in Japan to measure gene flow from *G. max* to *G. soja*. Gene flow was not detected. However, in populations of *G. soja*, the out-crossing rate ranged from 0 to 6.3% (Kuroda et al. 2008). The plant traits that contributed to insect pollinators were not studied in these reports.

The wild perennial relative of soybean, *G. argyrea* Tindale, has both self-fertilized cleistogamous flowers and chasmogamous flowers on the same plant (Brown et al. 1986). The chasmogamous flowers were visited by insect pollinators and ranged from zero to complete out-crossing, with an average of about 40%. *G. clandestina* (J. C Wendl.) is another perennial species closely related to *G. argyrea* with both cleistogamous and chasmogamous flowers (Schoen and Brown 1991). The floral biology of *G. clandestina* and *G. argyrea* allows chasmogamous flowers to spontaneously self-fertilize when left unpollinated; e.g., in the glasshouse and in the field when conditions for insect-mediated pollination are absent or suboptimal. Schoen and Brown (1991) sampled two populations of *G. clandestina* (1,500 and 750 m elevation) and one population of *G. argyrea*. In the 1,500 m population of *G. clandestina*, ~60% of the overall rate of self-pollination in



**Table 2** Comparison of floral nectary secretion and cell types among four legume taxa

Legume taxon	Secretory cells (SNP)	Type of secretion	Guard cells	Epidermal cells	Phloem cells	Gynoecium trichomes	References
<i>Glycine max</i>	Golgi, RER, mitochondria plastids, starch, vacuoles	Holocrine: degenerating vacuoles	Prominent; open; may be occluded; much starch	Similar to secretory cells; cuticle	Sieve tube elements Companion cells; parenchyma	Unicellular, non-secretory; 5–6 celled, secretory	Healy et al. (2005); Horner et al. (2003)
<i>Pisum sativum</i>	Isodiametric; starch; many small vacuoles; few organelles but many mitochondria	Ecrrine	Prominent; open; may be occluded; much starch	Similar to secretory cells; cuticle; many mitochondria	Sieve tube elements; Companion cells with wall ingrowths	Not mentioned	Razem and Davis (1999)
<i>Trifolium pratense</i>	Golgi; ER, mitochondria, Plastids, some starch; small	Ecrrine	Prominent	May be similar to secretory cells; cuticle	Not mentioned	Not mentioned	Eriksson (1977)
<i>Vicia faba</i>	Not mentioned	Granulo ecrrine	Prominent; open; may be occluded; much starch	Cuticle; wall ingrowths	Sieve tube elements	Not mentioned	Davis and Gunning (1992, 1993)

From Cervantes-Martinez et al. (2002)

SNP special nectary parenchyma cells, RER rough endoplasmic reticulum, ER endoplasmic reticulum

chasmogamous flowers was attributable to whole-flower selfing. This contrasts to the zero whole-flower selfing in chasmogamous flowers recorded with the 750 m population of *G. clandestina*. The difference in cross-pollination of the chasmogamous flowers between the two *G. clandestina* populations was considered to be related to contrasts in the environmental conditions for insect-mediated cross-pollination. The chasmogamous flowers that did not receive pollinators would self-fertilize spontaneously (Schoen and Brown 1991). In the *G. argyrea* population, only about 4% of the chasmogamous self-pollination was attributable to whole-flower selfing (Schoen and Brown 1991). Hempel (2004) has identified insect pollinators that contributed to out-crossed seed set on *G. clandestina* in Canberra and Namadgi NP in Australia as the introduced honeybee and native insects in the genera *Lasioglossum*, *Leioproctus*, and *Trichocolletes*.

#### *Cultivated species*

Early reports on out-crossing in soybean were done with caged plots. Values ranged from as low as 2% (Gumisiriza and Rubaihayo 1978) to as high as 60% of the fertile plant seed set (Roumet 1992; Roumet and Magnier 1993). In field plots, May and Wilcox (1986) examined the effect of pollinator density on frequency of contribution of pollen donors, ie pollen preference by natural insect pollinators. Differences in the slope of response between the two pollinators were interpreted as preferences by the insect pollinators.

A review of the natural cross-pollination literature in the cultivated soybean was given by Palmer et al. (2001). Since that review, Ray et al. (2003) conducted out-crossing studies with different soybean cultivars and planting patterns and reported cross-pollination rates from 0.7 to 6.3%. Their report of 6.3% is atypically high for soybean cultivars.

Chiang and Kiang (1987) studied out-crossing rates on three soybean cultivars with two different flower colour combinations and three geometrically different planting arrangements for 4 years. The average out-crossing rate was significantly higher in the mixed genotype planting than in the pure planting. Also the planting with a single flower colour had a significantly higher out-crossing rate than the plantings with mixed flower colours.

Ortiz-Perez et al. (2008a) compared honeybees and alfalfa leaf cutting bees at two locations for 3 years using male-sterile, female-fertile soybean genotypes. Neither the effect of pollinator species nor the interaction effect of pollinator species  $\times$  location was significant for any year for seed-set. These results are in contrast to Abrams et al. (1978) who observed a 60% reduction in alfalfa leaf cutting bee numbers and minimum visitation to soybean flowers when compared to honey bees.

#### *Insect-mediated cross-pollination for population improvement*

Population improvement in soybean can be facilitated with the use of male sterility to generate hybrids (Brim and Stuber 1973; Lewers and Palmer 1997). The segregation of genetic male sterility within a population offers a means for producing seed. Removal of the male-fertile siblings before flowering in the line being used as the female parent results in hybrid seed from insect-mediated cross-pollination.

Lewers et al. (1996) evaluated three methods to produce hybrid soybean seed; the traditional method, the dilution method, and the co-segregation method. With the traditional method, fertile siblings are removed at flowering. This method requires a substantial amount of time during flowering to remove the fertile siblings. The dilution method (Graef and Specht 1992) combines seed of a desired pollen parent with seed segregating for the desired male-sterile, female-fertile parent. The major advantage of the dilution method is that no rouging of fertile plants is required. This method requires a considerable amount of land and pollen-parent seed. The co-segregation method takes advantage of the close genetic linkage between the  $W_1$  locus (flower colour), and the  $Ms_6$  locus (pollen fertility; Palmer and Skorupska 1990). In addition, the  $W_1$  locus has a pleiotropic effect on hypocotyl colour. Thus, purple hypocotyl colour phenotypes can be removed at the seedling stage. The white-flowered plants are male sterile, except for any white-flower, male-fertile recombinants which can be identified and removed at flowering. Data from three locations, 2 years, and three replications per location indicated that the co-segregation method produced more insect-mediated hybrid seed per male-sterile plant than did the other two methods (Lewers et al. 1996).

St. Martin and Ehounou (1989) used insect-mediated cross-pollination and monitored pollinations of male-sterile plants of a soybean population to evaluate equality of male-parent contribution. Eight pollen parents were used with two replications for 3 years. Statistically significant differences among pollen-parent contributions were detected in all 3 years. Differences in pollinations of male-sterile, female-fertile plants could not be accounted for by the distance between parents. Honeybee visitations were observed but not quantified.

Lee et al. (1992) reported that random mating using insect pollinators was not observed for all traits in an intermating population of 39 female parents that varied in four morphological traits and in chloroplast DNA patterns. After 7 cycles of intermating (out-crossing), plants were scored for the four traits and chloroplast DNA patterns. The population underwent random mating with respect to flower colour and pubescence colour, but not in respect to seed colour, pubescence type, or chloroplast DNA pattern.

In an analysis of cytoplasmic diversity in an out-crossing population that relied on insects visiting male-sterile plants, Lee et al. (1994) found that four of the six cytoplasmic types (chloroplast DNA and mitochondrial DNA) that founded the population were lost after 7 cycles of seed increase. Four of the six cytoplasmic patterns were lost after only 1 cycle. The authors did not determine the reason(s) for this shift. Possible explanations included differences in plant height, duration of flowering period, or maturity. The insect pollinators were not monitored.

*Glycine max* soybean germplasm has been identified that shows variation to insect pollinators in Iowa and Texas (USA). The magnitude of attraction/reward, as measured indirectly by out-crossed seed set, varied according to location but the rank order was similar across pollinators (Ortiz-Perez 2005). The use of phenotypic recurrent selection, in a favourable environment, was successful in increasing the number of insect-mediated cross-pollinated seeds per male-sterile, female-fertile plant. Selection was based on the rationale of allowing insect pollinators to select the plants (female and male) that was then reflected in an increase in cross-pollinated seed set.

Figure 1 shows the steps that were used to advance the germplasm in the phenotypic recurrent selection procedure. Note that a sample of the  $F_1$  seed from each cycle was used for agronomic performance tests.

Because of the tremendous labour effort to rogue the male-fertile siblings in each female row, it was not possible to have each cycle synthesized each year for a comparative evaluation (Ortiz-Perez et al. 2008b). One experiment used backcrossing and another experiment introduced new male parents in the phenotypic recurrent selection study. Both procedures resulted in numerical increases in the number of cross-pollinated seed per male-sterile plant. The result was that three backcrosses (Table 3) or the introduction of four high yielding male parents (Table 4) increased seed-set on male-sterile plants as the result of insect-mediated cross-pollination.

The next step is to develop pairs of near-isogenic lines (for high and low out-crossed seed set) and measure plant traits in an attempt to determine genetic factors that determine pollinator attraction/reward or pollinator repulsion. Traits to be monitored include flower structure, flower orientation on the plant, number of open flowers per plant per day, and nectar quantity and composition. Pollinator visitations will be recorded and pollinators will be taxonomically identified.

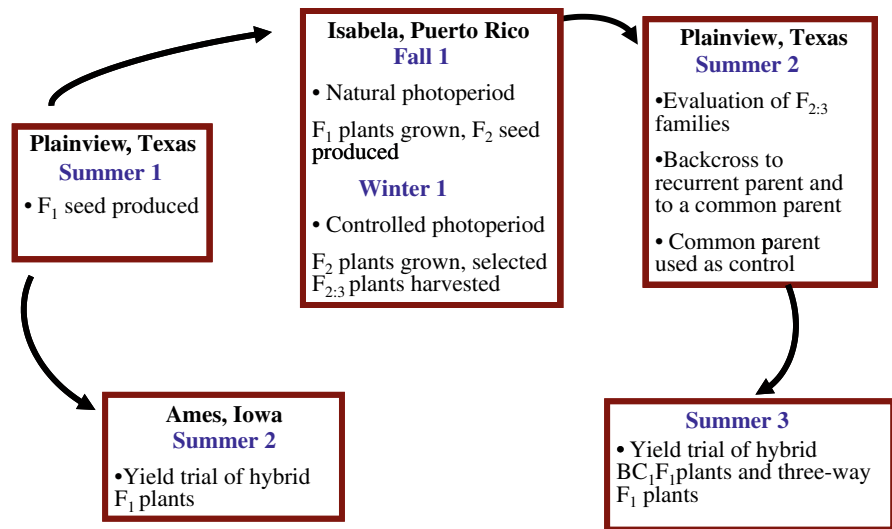
### *Vicia faba* L.

*Vicia faba* is particularly suitable for the application of CPR knowledge for at least two reasons: (1) it has been considered as partially allogamous in most genetic and breeding studies carried out to date (Link 1990; Carre et al. 1993; Link et al. 1994; Suso and Moreno 1999; Suso et al. 2001; Gasim et al. 2004); (2) it contains considerable floral variation associated with mating systems (Suso et al. 2005a).

Its mating system has been examined by using different approaches, both single and multilocus; and tools, including morphological and molecular markers. Studies on faba bean have been focused on the analysis of out-crossing within cultivars (Suso et al. 2001), and pollen-mediated gene flow between cultivars. Breeding questions include the choice of appropriate procedures for cultivar development that exploit heterosis and maximize yield stability (Metz et al. 1993, 1994; Pierre et al. 1996; Carre et al. 1998; Suso et al. 2005a), and for maintenance of varietal and germplasm purity during seed multiplication (Suso et al. 2008).

Faba bean plants simultaneously display multiple flowers, so that interaction with pollinators, which

**Fig. 1** Seed set production on male-sterile female-fertile soybean lines



**Table 3** Seed-set from fertile female soybean parents-derived BC<sub>3</sub> crosses compared in percent relative to their fertile female parent (Texas 2005)

Fertile female parent	Mean no. seed/fertile-female parent	Fertile female parents-derived BC <sub>3</sub> crosses*	Mean no. seed/male-sterile line	% Seed-set relative to fertile female parent
A00-39 <i>Ms2</i>	217	A00-39 <i>ms2</i> × Corsoy 79	91	42
A00-39 <i>Ms2</i>	217	A00-39 <i>ms2</i> × Hark	150	69
A00-41 <i>Ms2</i>	219	A00-41 <i>ms2</i> × A00-73 ( <i>Ms9</i> )	136	42
A00-63 <i>Ms2</i> (Beeson)	231	A00-63 <i>ms2</i> × Wells	99	43
A00-68 <i>Ms3</i>	287	A00-68 <i>ms3</i> × A00-41 ( <i>Ms2</i> )	232	80
A00-73 <i>Ms9</i>	384	A00-73 <i>ms9</i> × Raiden	217	56
A94-20 × 19 ( <i>Ms6</i> )	281	A94-20 × 19 ( <i>ms6</i> ) × A00-39 ( <i>Ms2</i> )	170	60
Mean	244		146	
LSD	90		36	
CV	50		40	

\* Ortiz-Perez et al. (2008b). With kind permission of Springer Science and Business Media

determines their level of out-crossing, can vary with both individual floral traits and the aggregate properties of the entire floral display, especially the number of open flowers (flower display and size). An important line of inquiry seeks to relate flower and inflorescence traits (floral morphology, structure, rewards and advertisement, inflorescence distribution in time and space) to observed levels of out-crossing (Suso et al. 2005a). These floral traits can then be the target of future selection for the development of open-pollinated varieties that maintain heterozygosity and optimise CPR. More specifically, Suso et al. (2005a) addressed relationships between levels of out-crossing

and floral phenology, design and display in two synthetic populations under open-pollination conditions with the indigenous, solitary bee *Eucera numida* Lep. serving as the principal pollinator (Pierre et al. 1999). The multilocus out-crossing rate was estimated under the mixed-mating model (MMM) of Fyfe and Bailey (1951) by analysis of progeny arrays based on levels of genetic polymorphism at seven allozyme markers and the MLTR program of Ritland (2002). Multilocus estimates of out-crossing rates confirmed the mixed mating system. Multivariate regression analysis revealed the strong influence of floral traits on the level of out-crossing and that floral

**Table 4** Seed-set from fertile female soybean parents-derived five-way crosses compared in percent relative to their fertile female parent (Texas 2005)

Fertile female parent	Mean no. seed/fertile-female parent	Fertile female parents-derived five-way crosses*	Mean no. seed/male-sterile line	% Seed-set relative to fertile female parent
A00-39 <i>Ms2</i>	217	A00-39 <i>ms2</i> × Corsoy 79	168	77
A00-39 <i>Ms2</i>	217	A00-39 <i>ms2</i> × Hark	164	75
A00-41 <i>Ms2</i>	219	A00-41 <i>ms2</i> × A00-73 ( <i>Ms9</i> )	217	99
A00-63 <i>Ms2</i> (Beeson)	231	A00-63 <i>ms2</i> × Wells	137	59
A00-68 <i>Ms3</i>	287	A00-68 <i>ms3</i> × A00-41 ( <i>Ms2</i> )	234	81
A00-73 <i>Ms9</i>	384	A00-73 <i>ms9</i> × Raiden	242	63
A94-20 × 19 ( <i>Ms6</i> )	281	A94-20 × 19 ( <i>ms6</i> ) × A00-39 ( <i>Ms2</i> )	124	44
Mean	244		183	
LSD	90		57	
CV	50		50	

\* Male 1, DSR Experimental 202b; Male 2, GH 4190; Male 3, DSR Experimental 202c

design and display traits affected differences in out-crossing unequally, with most variation being associated with the numbers of displayed flowers and inflorescences. Variation among plants in reward traits and in shape had limited and inconsistent influences on out-crossing. Flowers with more nectar do not necessarily increase the level of allogamy. Overall, the results implied that out-crossing might be enhanced by selection for plants that produce more inflorescences, each with relatively few flowers. Plants with low reward and short floral tubes should also promote out-crossing. Selection for decreased investment in nectar production may also allow plants to reallocate resources to ovules, providing an additional benefit towards seed production.

Pollen-mediated gene flow also can have a strong impact on seed-stock multiplications. Suso et al. (2008) attempted to identify floral traits associated with pollen-mediated gene flow and was designed to prevent contamination by pollen from neighbouring germplasm accessions. These CPR traits might then be used as predictors of potential gene flow to improve between-plot isolation strategies in the field for seed multiplication. Three isolation strategies were tested: (a) a barren zone, an isolation zone devoid of all vegetation; (b) the same size isolation zone sown with two different trap crops: (1) a faba bean male-sterile variety; and (2) a tetraploid genotype; and (c) the same size isolation zone sown with non-pollinated crop, a *Vicia narbonensis* population. The male-sterile variety does not release viable pollen, and the

tetraploid genotype does not cross with diploid faba bean genotypes. Consequently, the trap crops were used as pollen sink for bees to deposit pollen as they moved away from the test plots. *V. narbonensis* was used because of its similarity to *V. faba*; but because it is an autogamous crop, it would discourage insect pollinators from leaving the faba bean plots. Four genotypes, fixed for alternative isozyme alleles, allowed identification of between-plot hybrids through progeny testing. Paternity analysis and multivariate regression models showed that pollen-mediated gene flow is largely dependent on floral traits, confirming their utility for generating hypotheses about cultivar out-crossing or inbreeding behaviour. This can help provide more efficient procedures for seed-stock multiplication. Floral advertisement seemed to be important in explaining gene flow between plots surrounded by a barren zone. With regard to plots surrounded by a *V. narbonensis* population, the role of a reward trait, pollen production, was established. In contrast, in plots surrounded by faba bean trap crops, ovary length played the most important and consistent role in accounting for variation in gene flow.

Conclusions drawn from both studies (Suso et al 2005a, 2008) should be considered preliminary because out-crossing and pollen-mediated gene flow may vary widely geographically (Link et al. 1994; Suso and Moreno 1999) depending on local environmental conditions, particularly the composition of the pollinator fauna (Bond and Kirby 1999, 2001; Pierre

et al. 1996, 1999). Thus, further assessment will be needed (Duc et al. 2008).

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